Neurochemical features of the autonomic neurons projecting to the cremaster muscle of the boar

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Neurochemical Features of the Autonomic Neurons Projecting to the Cremaster Muscle of the Boar

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ABSTRACT

The cremaster muscle (CM) is a striated muscle showing some unusual features for ordinary striated muscles, in fact it receives, besides somatic innervation, a conspicuous autonomic sympathetic innervation. The autonomic neurons associated with the CM of 4 male intact pigs were typified combining the retrograde nontrans-synaptic fluorescent tracer Fast Blue (FB) and double labeling immunohistochemical methods. We collected the L4 sympathetic trunk ganglion (STG), that our preliminary studies proved to contain the highest number (575.5 ± 152.93; mean ± S.E.M., $n = 4$) of FB+ sympathetic neurons projecting to CM. About half of the CM projecting neurons of this ganglion were catecholaminergic and showed the colocalization of Tyrosine Hydroxylase (TH) with Neuropeptide Y (NPY), Leu-Enkephaline (LENK), Vasoactive Intestinal Polypeptide (VIP), Calcitonine Gene Related Peptide (CGRP), Substance P (SP), neuronal Nitric Oxyde Sinthase (n-NOS), and Vesicular Acetylcholine Transporter (VAChT). The noncatecholaminergic neurons were immunoreactive for all the other markers tested, even if in small percentages. The conspicuous and heterogeneous contribution of the sympathetic autonomic neurons to the muscle innervation is consistent with the hypothesis of a possible origin of the CM fibers by transdifferentiation of the smooth muscle-like gubernaculum mesenchyma into striated myotubes, suggesting that the cremaster myogenesis is independent from that of the abdominal muscles. Anat Rec, 00:000–000, 2015.

Key words: cremaster muscle; boar; immunohistochemistry; neuronal tracer; sympathetic innervation

The striated cremaster muscle (CM) shrouds the testis and moves the gonad up or down in response to the change of temperature. It extends from the iliac to the spermatic fascia occupying its dorsal edge and its lateral side (Barone, 1983; Dyce et al., 2002).

Thanks to its easy accessibility, the CM has been widely used in anatomical, physiological and pharmacological research in rat and humans (Franken et al., 1996; Berg et al., 1997; Tanyel et al., 2000; 2001a,b,c; Kobayashi and Takizawa, 2002; Zempoalteca et al., 2002; Tanyel et al., 2003; Ertekin et al., 2005) to explain the characteristics of its blood circulation (Anderson et al., 1988; Franken et al., 1996; Hungerford et al., 2000; Kobayashi and Takizawa, 2002; Ozmen et al., 2008; Mauban et al., 2013; Ngo et al., 2013) and innervation.

Concerning its innervation, like the striated musculature of esophagus and urethra, the CM does not require only a voluntary control (Ertekin et al., 2005; Tanyel

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et al., 2005), but receives also, in addition to somatic, an abundant autonomic innervation (Samarasinghe, 1972; Elbadawi and Schenk, 1974; Tanyel et al., 2001b; Botti et al., 2006 a; 2014). In agreement with this possibility, failed testis descent in boys has revealed a neurological damage of the CM resulting from a defect in autonomic nervous supply, which mainly involves the sympathetic division (Tanyel et al., 2002; Duman et al., 2010).

Until now, it was assumed that the CM derived from fibers of internal abdominal oblique muscle, but there is new positive evidence that the cremaster myogenesis is independent from that of the abdominal muscles. The muscle fibers, rather, seem to develop initially from primitive mesenchyma of gubernaculum (Sanders et al., 2011) probably transdifferentiating from its vascular smooth musculature (Patapoutian et al., 1995; Tanyel et al., 2005). A similar hypothesis has been formulated also for the human urethral muscle (Borirakchanyavat et al., 1997), as its developing myotubes coexpress both smooth and striated muscle markers.

As the majority of the neurons supplying the swine CM are autonomic and localized in the sympathetic trunk ganglia (STG) (Botti et al., 2006a), we wanted to study their neurochemical characteristics. The experimental animal used was the pig, because it is an important model for biomedical, neuro-anatomical, and zootechnical studies (Dodds, 1982; Swindle et al., 1992; Kaleczyc et al., 2002; Russo et al., 2013).

MATERIALS AND METHODS

All the procedures described below were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Italian legislation regarding experimental animals, after approval by the Scientific Ethics Committee for Experiments on Animals of the University of Parma (Prot. Rif. 19/04). All possible efforts were made to minimize the number of animals used and their suffering.

Four intact male crossbreed (Large white x Landrace x Duroc) pigs (aged 3 months, range body weight: 30 – 40 kg) were maintained on a diet and water ad libitum for 1 week before the experiment.

For 24 h prior to surgery, the animals were not given any food and preventive antibiotic therapy with Ceftiofur (Naxcel 5 mg/kg i.m., Pfizer, Sandwich Kent, UK) was administered. The animals were sedated by intramuscular injection of azaperone (4 – 10 mg/10 kg i.m., Stresnil, Janssen Cilag S.p.A., Cologno Monzese, Italy) and ketamine (150 mg/10 kg i.m., Ketavet 100, Intervet Italia s.r.l., Aprilia, Italy). After recumbency and venous catheterization (*vena auricularis caudalis*), anaesthesia was induced by the administration of propofol (2 – 6 mg/Kg i.v., Rapinovet; Schering Plough, Segrate, Italy) and maintained with 1.5% to 2% isofluorane in 100% oxygen delivered by an open circuit, via a cuffed Magill orotracheal tube. The post-operative anti-inflammatory effect was achieved by an intramuscular injection of tolfenamic acid (2 mg/kg i.m., Tolfedine CS, Vétoquinol s.r.l., Italy), daily (for 5 days) postoperatively.

The pigs were placed in dorsal recumbency and an incision of the scrotum and external spermatic fascia was performed to visualize the left testis and the corresponding CM, where 100 μL of fluorescent tracer Fast Blue (FB; Sigma-Aldrich Chemie, Steinheim, Germany; 2% aqueous solution) were injected, by means of a manually operating glass Hamilton microsyringe. Injection sites were recognizable by observing the tracer's deposit beneath the cremasteric fascia. To avoid leakage, the needle was left in place for 1 min. The site of injections was then rinsed with physiological saline and gently wiped with gauze. The external spermatic fascia and the scrotum were closed using a routine three-layer suture procedure. The recovery and post-operative periods were uneventful. One week after the surgery, the pigs were deeply sedated and anaesthetized and intracardially perfused, first with heparinized physiological solution and afterward with 4% w/v of paraformaldehyde in phosphate buffer (0.1 M, pH 7.4).

The animals used in this study had 14 thoracic (Th), 7 lumbar (L), 4 sacral (S), and 20 caudal (Ca) vertebrae.

Specificity of the Injection Sites

Macroscopic and microscopic examinations of the sites of FB injections were performed. Macroscopically, the tracer left a yellow-labeled deposition at the injections sites. To verify that the tracer had not migrated into the testis, we analyzed, the part of the testis surrounded by the CM, using fluorescent microscopy of cryosections. In all four subjects the tracer was confined within the striated musculature of the CM.

Tissue Preparation

From each subject we collected the left L4 STG, that we have documented to contain the highest number of autonomic neurons projecting to the pig CM (Botti et al., 2006a). The samples were post-fixed (paraformaldehyde in phosphate buffer) and serially cut in 16 μm thick sections along their longest axis, in order to obtain a large number of cells per section.

Immunohistochemistry

The sections were stained by a double labeling immunofluorescence method to test the colocalization of Tyrosine Hydroxylase (TH) with Calcitonin Gene-Related Peptide (CGRP), Leu-Enkephalin (LENK), neuronal Nitric Oxyde Synthase (nNOS), Neuropeptide Y (NPY), Substance P (SP), Vesicular Acetylcholine Transporter (VAChT), and Vasoactive Intestinal Polypeptide (VIP), and the eventual presence of each of these markers alone. Primary antisera, secondary reagents, and dilutions used are listed in Table 1. T1

The same combinations of primary antisera were applied to sections at least 112 μm away from each other to eliminate the likelihood of testing the same neuron twice for the same antisera. The sections were processed and observed according to the methods previously described in Ragionieri et al. (2013). The number of FB+ neurons was calculated by counting, in the sections, only the cells in which the nucleus was recognizable. The number of FB+ neurons was calculated by applying Abercrombie's formula (Guillery, 2002). The correction factor utilizes the T/T+h ratio, where T = section thickness (16 μm) and h = mean diameter of the sympathetic ganglion cells nuclei along the perpendicular axis to the plane of the section. The mean diameter of the nuclei of 100 sympathetic ganglion cells, evaluated on H&E stained sections, was 11.47 ± 1.3 μm. The correction factor was 0.58.

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TABLE 1. Primary antisera, secondary reagents and dilutions used

Primary antibody	Raised in	Code no.	Dilution	Supplier
Anti TH	Mouse (monoclonal)	T 2928	1:4000	Sigma, St. Louis, Missouri, USA
Anti CGRP	Rabbit (polyclonal)	C 8198	1:4000	Sigma, St. Louis, Missouri, USA
Anti LENK	Rabbit (polyclonal)	L 8516	1:5	Sigma, St. Louis, Missouri, USA
Anti n-NOS	Rabbit (polyclonal)	AB 5380	1:1500	Chemicon International, Inc., Temecula, CA
Anti NPY	Rabbit (polyclonal)	N 9528	1:4000	Sigma, St. Louis, Missouri, USA
Anti SP	Rabbit (polyclonal)	S 1542	1:4000	Sigma, St. Louis, Missouri, USA
Anti VAChT	Rabbit (polyclonal)	V 5387	1:500	Sigma, St. Louis, Missouri, USA
Anti VIP	Rabbit (polyclonal)	V 3508	1:4000	Sigma, St. Louis, Missouri, USA
Secondary reagents				
Anti rabbit IgG/FITC	Goat	F 0382	1:40	Sigma, St. Louis, Missouri, USA
Anti mouse IgG/Biotin	Sheep	RPN 1001	1:100	Amersham Pharmacia Biotech Sweden
Streptavidin/Texas Red		RPN 1233	1:100	Amersham Pharmacia Biotech Sweden
Normal serum				
Goat serum		G 9023		Sigma, St. Louis, Missouri, USA

TABLE 2. Percentages of immunoreactivity (mean ± standard error among the 4 animals) for the combinations of antibodies tested on Fast Blue positive neurons in the sympathetic trunk ganglion L4 of 4 male pigs

Tested antibodies	TH+/Ab+	TH+/Ab−	TH−/Ab+	TH−/Ab−	FB+ cells
TH-NPY	50.72 ± 2.8%	15.38 ± 2.55%	11.12 ± 4.32%	22.79 ± 2.78%	210 ± 53.2
TH-LENK	37 ± 4.25%	40.52 ± 5.96%	3.54 ± 1.37%	18.94 ± 2.09%	248.5 ± 73.82
TH-VIP	30.95 ± 8.2%	29.51 ± 3.87%	8.38 ± 2.33%	31.16 ± 6.04%	202.5 ± 47.65
TH-CGRP	29.4 ± 4.36%	29.09 ± 7.61%	11.45 ± 4.43%	30.06 ± 3.7%	213.25 ± 71.82
TH-SP	26.76 ± 4.73%	36.82 ± 7.58%	3.78 ± 2.43%	32.64 ± 3.75%	218.75 ± 65.02
TH-nNOS	24.72 ± 5.43%	29.35 ± 7.71%	7.23 ± 3.22%	38.7 ± 2.88%	211.5 ± 47
TH-VAChT	24.52 ± 5.6%	33.65 ± 7.33%	7.46 ± 2.77%	34.38 ± 2.66%	206 ± 51.07

The meaning of the abbreviations used for each antibody is given in the key to abbreviations.

Data are expressed as means ± SEM among the four animals.

Antibody Characterization

The specificity of the anti-CGRP (Sigma-Aldrich), anti-nNOS (Chemicon-Millipore), anti-TH (Sigma-Aldrich), anti-NPY (Sigma-Aldrich), and anti-VAChT (Sigma Aldrich) antibodies has been recently tested by Western blot (Wb) analysis on porcine tissues (Russo et al., 2013; Ragionieri et al., 2013). Furthermore, the Sigma-Aldrich or Amersham datasheets state the specificity of anti-SP, anti-VIP, anti rabbit IgG/FITC, anti mouse IgG/Biotin and Streptavidin/Texas Red antibodies for porcine tissues.

We tried to test unsuccessfully by Wb also the specificity of the polyclonal anti-LENK (Chemicon) antibody, although the Chemicon datasheet specified that the product is not usable for this application. However no immunoreactivity was detected in a control experiment that was done, incubating sections in the absence of primary antiserum replaced by PBS. LENK is an opiod peptide derived from the same mammalian precursor (Noda et al., 1982), thus it is reasonable to believe that the anti-LENK utilized in the present research recognizes the pig polypeptide.

RESULTS

In the studied ganglion, the relative number of FB+ neurons was 575.5 ± 152.93. The primary antisera, in each combination, were tested on a mean number of 215.78 ± 20.01 FB+ cells for every animal. The percentages of cells positive to FB coexpressing TH with the other markers tested or immunoreactive (-IR) for only one of the markers tested are reported in Table 2. T2

Approximately half of the FB + neurons showed immunoreactivity for TH, and therefore were of a catecholaminergic nature.

The FB+ catecholaminergic neurons coexpressed all the substances tested, in the following percentages: NPY- (50.72 ± 2.8%) (Fig. 1A−C), LENK- (37 ± 4.25%) (Fig. 1D−F), VIP- (30.95 ± 8.2%) (Fig. 1G−I), CGRP- (29.4 ± 4.36%) (Fig. 1L−N), SP- (26.76 ± 4.73%) (Fig. 1O−Q), nNOS- (24.72 ± 5.43%) (Fig. 1R−T), and VAChT-immunoreactivity (24.52 ± 5.6%) (Fig. 1U−Z). F1

The noncatecholaminergic (TH-immunonegative) neurons projecting to the CM showed positivity for all the other markers used: CGRP- (11.45 ± 4.43%), NPY- (11.12 ± 4.32%), VIP- (8.38 ± 2.33%), VAChT- (7.46 ± 2.77%), nNOS- (7.23 ± 3.22%), SP- (3.78 ± 2.43%), and LENK- (3.54 ± 1.37%).

DISCUSSION

Combining the retrograde neuronal tracer and double immunohistochemical labeling techniques, this research allowed us to demonstrate the different neurochemical characteristics of the sympathetic neurons innervating the boar striated CM.

TH-Immunoreactivity

The unexpected low percentage (about 50%) of immunoreactivity to TH found in the present study may be due to the age of the experimental animals used

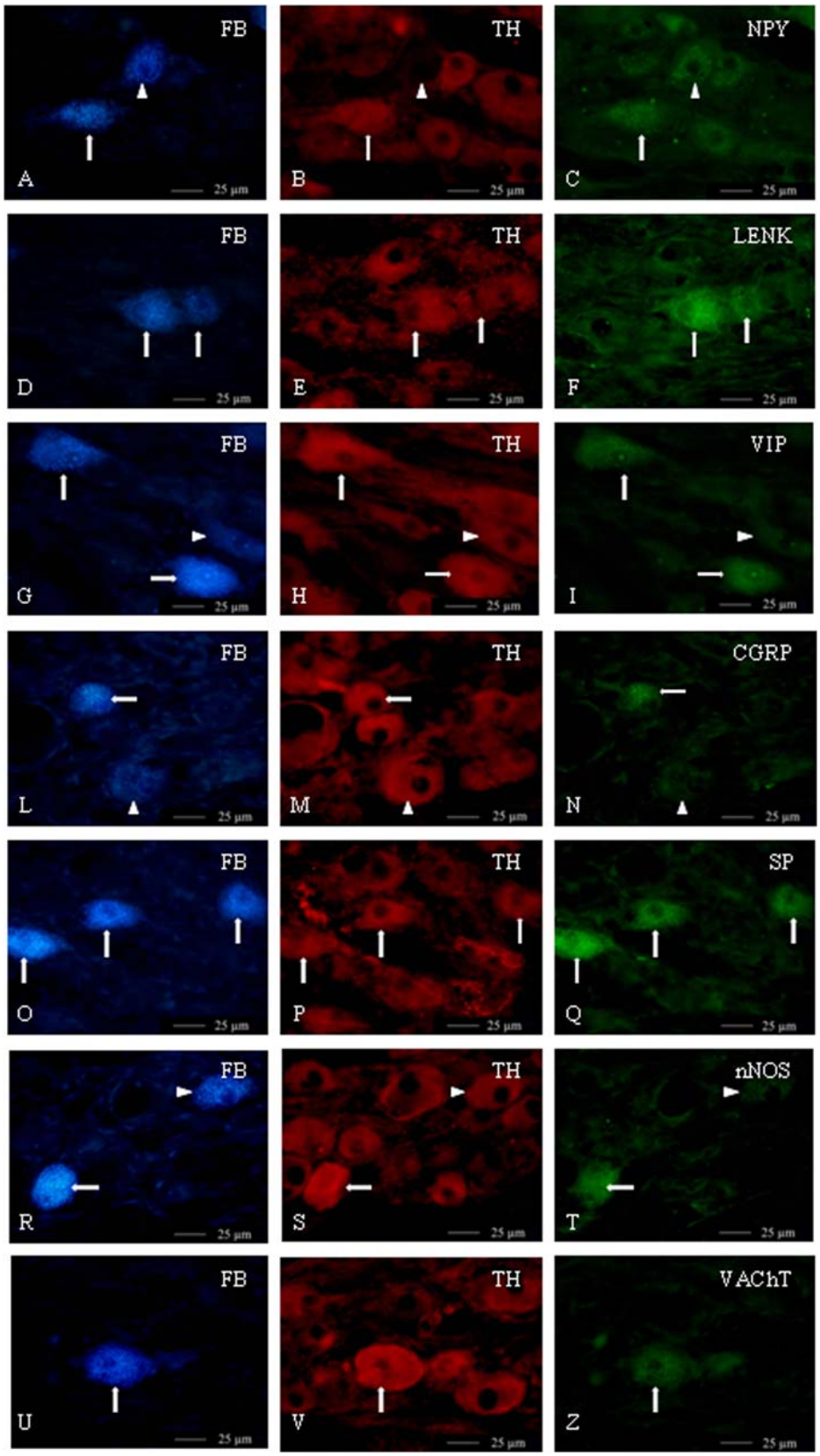

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Fig. 1. **A**–**Z**: The micrographs show multipolar neurons innervating the pig CM. (A, D, G, L, O, R, U): positivity to FB; (B, E, H, M, P, S, V): immunoreactivity for TH; (C, F, I, N, Q, T, Z): immunoreactivity for NPY, LENK, VIP, CGRP, SP, nNOS, VAChT. The arrow points out the FB+ neurons contemporaneously IR to TH and to one of the other tested markers. The arrowhead points out the FB+ neurons IR only to TH or one of the other tested markers.

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(Maslyukov et al., 2007) as the autonomic neurons projecting to genital organs are influenced by serum concentration of steroid hormones (Keast, 1999, 2000; Sienkiewicz, 2010). According to the data of Colenbrander et al., (1978), the 3-month-old pigs that we used could have low levels of serum testosterone and, for this reason, could have relatively small percentages of catecholaminergic neurons, if compared with percentages of catecholaminergic neurons projecting to the genital organs of sexually mature boars or sows (Sienkiewicz, 2010; Koszykowska et al., 2010).

As TH catalyzes the rate limiting step in the synthesis of catecholamines (noradrenaline and adrenaline) and these last are excitatory in nature causing contraction of smooth muscular cells (Keast, 1999; Andersson et al., 2000), the TH+ neurons, found in our study, could have a vasoconstrictor activity and they could be involved in gonadal thermoregulation. These neurons could also take part in muscle metabolism inducing a sympathetic vasoconstriction that increases and preserves the muscle local pressure (VanTeeffelen and Segal, 2003) after the initial functional vasodilation, occurring when the skeletal muscle starts its contraction, to draw oxygen and nutritional substances from the blood. (Sweeney and Sarelius, 1989; Van Teeffelen and Segal, 2000; Thomas and Segal, 2004).

NPY-Immunoreactivity

NPY is a polypeptide widespread in the central and peripheral nervous system, in particular in the postganglionic sympathetic neurons (Gazza et al., 2003; Botti et al., 2006b,,; Ragionieri et al., 2008), implicated in the modulation of the vasoconstrictor response triggered by catecholaminergic neurons. The NPY found in our study, in large percentages colocalized with TH, could be involved in blood flow regulation of the pig CM, increasing the effects of noradrenaline on the contraction of vasal musculature (Ekblad et al., 1984; Pernow et al., 1988). NPY could play its regulatory function also preventing, by a presynaptic mechanism, the release of acetylcholine from nervous endings (Lundberg et al., 1990).

LENK-Immunoreactivity

The sympathetic neurons innervating the pig CM were contemporaneously immunoreactive to LENK and TH in discrete percentage ($40.52 \pm 5.96\%$) and positive to LENK alone in a small percentage ($3.54 \pm 1.37\%$). This endogenous opioid neuropeptide seems to affect the autonomic neurotransmission with inhibitory action, showing a vasodilator effect (Konishi et al., 1981; Katayama and Nishi, 1984; Kaleczyc, 1988). The colocalization of enkephalins and TH in STG neurons (Konishi et al., 1981) could suggest that these peptides act at the neuro-effector junction, inhibiting sympathetic inputs (Klarskov, 1987; Czaja et al., 1996).

VIP-Immunoreactivity

A discrete percentage of the sympathetic catecholaminergic CM-projecting neurons were also positive for VIP, while a lower proportion was IR for VIP alone. The coexistence of TH and VIP, already documented by Hill and Elde (1989), was unusual because VIP is considered a marker of the cholinergic pathway. Nevertheless, these TH+/VIP+ STG neurons could have vasodilator effect. Also VIP-ergic noncatecholaminergic neurons could act on blood flow regulation, displaying a physiological antagonism with NPY (Morris et al., 1985), probably modulating the neuromuscular transmission.

CGRP-Immunoreactivity

CGRP is a 37-amino acid neuropeptide, a typical marker of sensitive pathways primarily released from afferent nerve fibers (Majewski et al., 1995; Czaja, 2000). In the STG, the CGRP immunoreactive neurons could modulate the neurotransmission on their target organs (Häppolä et al., 1993). In particular on the striated muscle, CGRP elicits contraction and increases the maximal contractile response (Tanyel et al., 2001c). Moreover, CGRP has been proposed to regulate inguinoscrotal testicular descent and to chemotactically guide the gubernaculum into the scrotum (Hutson, 1998; Lie and Hutson, 2011).

SP-Immunoreactivity

SP is a member of tachykinin family, mediating many biological actions both in central and peripheral nervous system. SP plays its physiological roles as a vasoactive and mitogenic factor (Mozaffari et al., 2015), changes the excitability of different neuronal subpopulations, modulates nociceptive stimulus (Moraes et al. 2014) and, like CGRP, is traditionally considered an afferent neurotransmitter. But it is also found in the autonomic neurons where it shows local effector functions (Tanyel et al., 2001c). Therefore, we maintain that SP could control the CM contraction or regulate the blood-flow in the muscle.

nNOS-Immunoreactivity

A discrete percentage of catecholaminergic cells showed immunoreactivity also for nNOS, while only 7% of sympathetic neurons projecting to CM contained this enzyme alone. nNOS is the enzyme synthetizing NO, that is a regulator of blood flow. Therefore, NO could act on the CM vessels exerting relaxing activity and vasodilator effects.

Moreover, we cannot exclude a NO direct action on the CM myocells, in fact it has been demonstrated that NO exerts a small inhibitory action on boys CM contractility (Tanyel et al., 2001c).

Moreover NO, colocalized with TH in postganglionic neurons, shows a modulation function, because it could increase TH action, via dependent or independent guanylate cyclase activity (Klimaschewski et al., 1996).

VAChT-Immunoreactivity

The IR for VAChT was found in a discrete percentage of catecholaminergic CM projecting neurons, while a small number of sympathetic neurons showed positivity for VAChT alone.

The coexistence of cholinergic and catecholaminergic markers could be due to the fact that some neurons, during fetal development, switch from transitory cathecholaminergic to cholinergic phenotype (Masliukov and Timmermans, 2004). If so, in the sympathetic neurons

observed in our study, the catecholamine marker might not be completely disappeared in respect to the development of the cholinergic marker. Moreover, the cholinergic sympathetic innervation might regulate the adrenergic neurotransmission, that is excitatory, by means a presynaptic modulation mechanism, as has been hypothesized by Creed and Van der Werf (2001) for the human urethral muscle.

CONCLUSIONS

In the present research, carried out combining the nontrans-synaptic tracer and double labeling immunohistochemical methods, we proved that approximately one-half of the sympathetic neurons innervating the blood vessels and the myofibers of the porcine CM are catecholaminergic in nature and contain different neuropeptides and neuromodulators colocalized with TH. The release of different substances seems to be the mechanism commonly employed by the sympathetic neurons to regulate and/or modulate the activity of the muscle. In fact, the percentages of FB+ neurons showing immunoreactivity only for the other substance tested in association with TH were always very small.

In conclusion, we think that the conspicuous and heterogeneous contribution of the sympathetic autonomic neurons to the muscle innervation is consistent with the hypothesis of a possible origin of the CM fibers by transdifferentiation of the smooth muscle-like gubernaculum mesenchyma into striated myotubes (Tanyel et al., 2005), suggesting that the cremaster myogenesis is independent from that of the abdominal muscles.

Further studies will be carried out to define the immunohistochemical characteristics of the prevertebral and spinal ganglia projecting to the CM.

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